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Short Communication

The Heavy Metal-Responsive Transcription Factor-1 (MTF-1) Is Not Required for Neural Differentiation

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The zinc finger transcription factor MTF-1 is essential for proper response to heavy metal load and other stress conditions in vertebrates, and also contributes to the maintenance of the cellular redox state. Target genes include metallothioneins (MT-I and MT-II) and gamma-glutamylcysteine synthetase (gamma-GCS), an enzyme involved in glutathione biosynthesis. Although MTF-1 is expressed ubiquitously, the primary defect in null mutant mice is hepatocyte necrosis, which results in embryonic lethality around day E14 and prevents the analysis of delayed effects on other organs. To assess the impact of MTF-1 deficiency on the function of the mature central nervous system, we employed the neural grafting strategy. Neuroectodermal brain tissue obtained from transgenic mouse embryos at gestational day 12.5 was transplanted into the caudoputamen of adult wild-type mice. 33 days later, grafts derived from MTF-1 deficient mice consisted of fully differentiated neuroectodermal tissue and showed no differences to heterozygous control grafts. This indicates that MTF-1 is dispensable for the development and differentiation of the nervous system. Such transplants devoid of MTF-1 may provide a useful tool for the further investigation of the effect of cell stress, including oxidative stress.

Key words: Cell stress / Metallothionein-III /
Neural development / Neural transplantation.

Most organisms have developed specialized defense mechanisms to sense and respond appropriately to environmental and physiological stresses, such as toxic metals, oxidative stress, heat shock and osmotic stress.

One class of free-radical scavengers important for coping with oxidative stress are small antioxidant molecules such as glutathione, thioredoxin and ascorbic acid (Chance *et al.*, 1979; Sies, 1986; Halliwell, 1994; Björn-

stedt *et al.*, 1997; Sies, 1997). Another class of eukaryotic stress responsive molecules are metallothioneins (MTs). MTs are small, cysteine-rich metal-binding proteins whose biosynthesis is induced predominantly at the transcriptional level by a variety of environmental and physiological stressors such as heavy metals and reactive oxygen species (ROS) (Kägi and Vallee, 1960; Thornally and Vasak, 1985; Angel *et al.*, 1986; Kägi, 1991).

The metal-regulatory transcription factor 1 (MTF-1) has been shown to be crucial for metallothionein I and II gene expression. MTF-1 is a highly conserved, ubiquitously expressed zinc finger transcriptional activator, and has been characterized in detail (Westin and Schaffner, 1988; Labbé *et al.*, 1993; Radtke *et al.*, 1993; Heuchel *et al.*, 1994; Otsuka *et al.*, 1994; Bittel *et al.*, 1998). MTF-1 exerts its activity by binding to a number of so-called metal responsive elements (MREs), present in the promoters of metallothionein I and II genes and a number of other genes (Stuart *et al.*, 1984; Searle *et al.*, 1985; Heuchel *et al.*, 1994; Günes *et al.*, 1998). In addition, the recent characterization of MTF-1 from the Japanese pufferfish (*Fugu rubripes*) revealed a structural and functional conservation of this protein over 400 million years of evolution, supporting the notion that MTF-1 is an important eukaryotic stress sensor (Auf der Maur *et al.*, 1999).

We recently generated MTF-1 null-mutant mice by targeted gene disruption (Günes *et al.*, 1998). Mice lacking MTF-1 die *in utero* at about day 14 of gestation as a result of liver cell necrosis, while heterozygous mice develop normally and do not show any abnormalities under laboratory conditions. Primary cells derived from MTF-1^{-/-} embryos show an increased sensitivity to cadmium and hydrogen peroxide. Since a combined knockout of MT-I and MT-II genes is viable (Masters *et al.*, 1994a), we looked for further target genes of MTF-1 and found the gamma-glutamylcysteine synthetase heavy chain (gamma-GCS^{hc}), a subunit of a key enzyme involved in the biosynthesis of glutathione, to be downregulated in MTF-1 knockout embryos (Günes *et al.*, 1998). Because glutathione is a major contributor to the cellular defense against ROS (Halliwell, 1994; Meister, 1995), it seemed to be likely that elimination of the ubiquitously expressed MTF-1 may also have adverse effects on tissues other than the liver.

While MT-I and II are ubiquitously expressed, a third member of metallothioneins, MT-III is expressed only in a specific subset of the neuronal population within the central nervous system (CNS). These neurons are mainly glutamatergic hippocampal neurons (pyramidal neurons and dentate granule cells), that release zinc from their synaptic

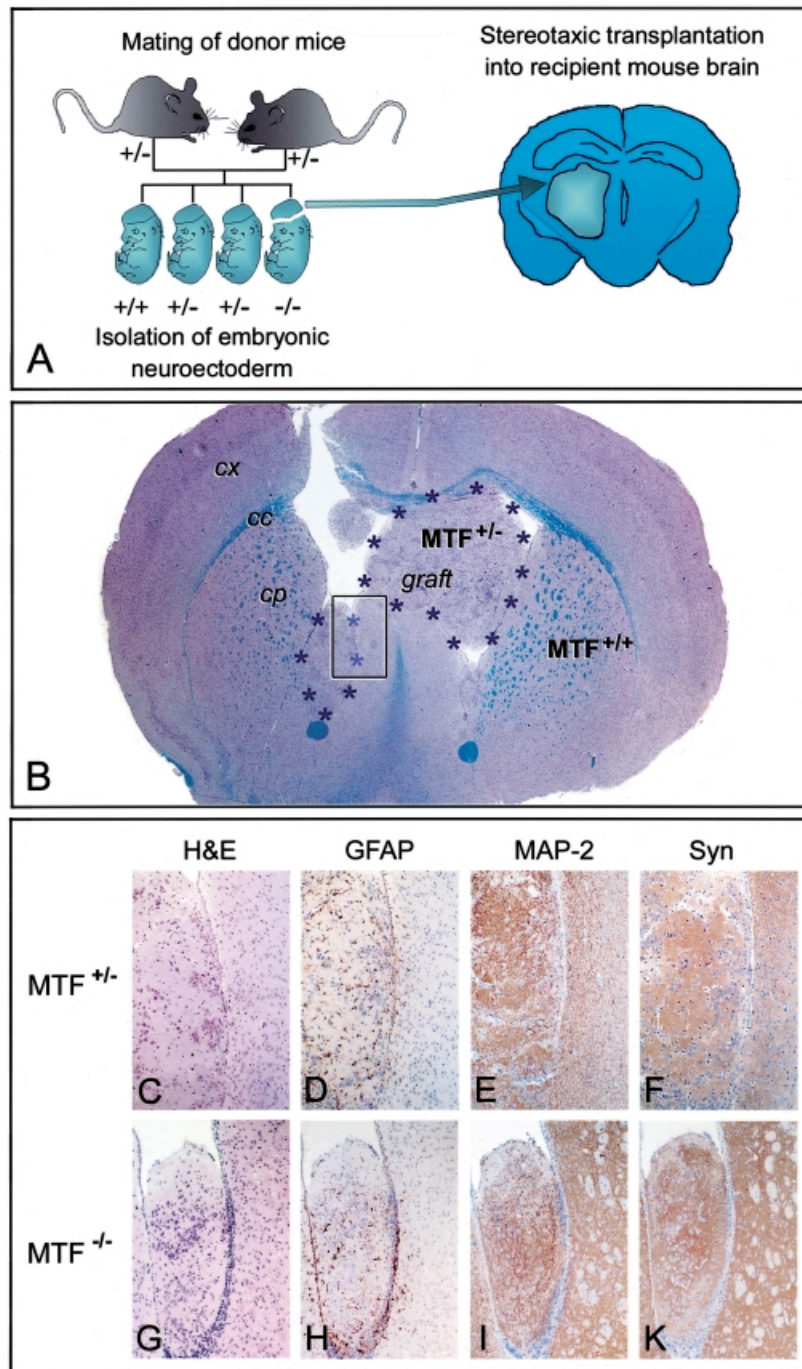


Fig. 1 Immunohistological Analysis of Brain Tissue after Neuroectodermal Grafting in Mice.

(A) Schematic drawing of the neuroectodermal grafting procedure: mice heterozygous for MTF-1 were mated, and progeny embryos were removed at embryonic day 12.5. Neuroectoderm was removed under a dissection microscope and then stereotactically injected into the caudoputamen region of adult recipient mice.

(B) Low-power magnification of a frontal section of a mouse brain containing two large portions of a MTF^{+/-} neural graft in the ventricular system (delineated by asterisks). The box indicates the area shown at high magnification in Figure 1C-F. Cx, cortex; cc, corpus callosum; cp, caudoputamen. Hematoxylin-eosin (H&E) combined with luxol fast blue.

(C-K) High-power magnification of intraventricular neuroectodermal MTF-1^{+/-} and MTF-1^{-/-} grafts 33 days after implantation. (C) The H&E stained section shows the localization of the graft in the ventricular system. GFAP immunostain (D) reveals the presence of astrocytes, which are more abundant than in the surrounding host tissue. Immunostains for the neuronal markers MAP-2 (E) and synaptophysin (F) show the same density and appearance of dendrites and synapses in graft and host tissue. The comparison with MTF-1^{-/-} grafts (G-K) shows essentially the same findings. In particular, there is no difference in the expression pattern of astrocytes and neurons.

Methods: mating of mice: mice heterozygous for MTF-1 were mated and the morning at which a vaginal plug was detected was defined as embryonic day E0.5. The embryos were harvested at day E12.5, and the neuroectodermal tissue was dissected and implanted into the caudoputamen of anesthetized six week-old wild-type female mice (C57 BL/6), using a stereotaxic frame and coordinates as previously described (Isenmann *et al.*, 1995).

terminals (Masters *et al.*, 1994b). MT-III may be an important regulator of zinc in the CNS, in particular during neural stimulation, since MT-III-deficient mice have decreased concentrations of zinc in specific brain regions and are more susceptible to kainic acid-induced seizures (Erickson *et al.*, 1997). The main target of neuronal injury is the CA3 field of the hippocampus (Erickson *et al.*, 1997). MT-III is most abundantly expressed in zinc-containing neurons, binds zinc *in vivo* (Masters *et al.*, 1994b), and confers resistance to zinc cytotoxicity in cultured cells (Palmiter 1995).

While the role of factors acting downstream of MTF-1 (*i.e.* MT-I, MT-II and potentially MT-III) in CNS function has been elucidated to a considerable extent by the previously mentioned studies, it is unclear what role MTF-1 plays for the development and function of the mature CNS, as mice devoid of MTF-1 show midgestation lethality due to liver cell necrosis. We therefore devised a nontransgenic approach aimed at the rescue of MTF-1-deficient central nervous system tissue. The main questions addressed were whether MTF-1-deficient CNS tissue is viable at all, and if so, whether there are tissue-specific adverse effects due to an increased sensitivity to oxidative stress caused by a greatly reduced synthesis of gamma-GCS_{nc}, and a possible subsequent decrease of glutathione levels in the affected tissue. Furthermore, we wondered whether a possible MTF-1-dependent reduction of MT-III levels (in addition to the lack of MT-I and MT-II in MTF-1^{-/-} neural tissue) leads to a disturbance in zinc metabolism, and thereby to a potential, zinc-induced neurotoxicity.

In our experimental design, embryonic lethality caused by selective hepatocellular failure at embryonic day 14 was circumvented by explanting viable embryos at day 12.5. At this gestational age, the neuroectodermal brain anlage is immature, but already committed to further differentiation into neuronal and glial cells. Transplantation of such neuroectodermal stem cells into the brain of an adult wild-type mouse has been shown to faithfully recapitulate the neural differentiation and lead to mature, fully-differentiated graft tissue (Isenmann *et al.*, 1995, 1996; Brandner *et al.*, 1996). Transplantation of neural tissue was therefore performed essentially as described (Isenmann *et al.*, 1995, 1996). (Figure 1A, B).

After implantation, the remaining embryo tissue was subjected to PCR genotyping as described previously (Günes *et al.*, 1998). Neural tissue from nine embryos (three MTF-1^{-/-} and six MTF-1^{+/-}; no wild type) was transplanted into the caudoputamen of nine recipient mice. All graft recipients were kept under normal laboratory conditions in

the animal facility. They were clinically monitored every other day for development of neurological deficiencies, and were sacrificed for histological analysis after 33 days. Analysis comprised hematoxylin-eosin (H&E), luxol fast blue, and immunohistochemical stains for neuronal and glial antigens (Figure 1 C-K). By morphological appearance, viable grafts were detected in all recipient mice. Grafts of both genotypes (MTF-1^{-/-} and MTF-1^{+/-}) were indistinguishable. They differentiated into neuroectodermal tissue with regular neuropil structure, showed normal morphological appearance of neurons and astrocytes, and exhibited no obvious abnormalities in their integration into the host tissue context. Immunostains for astrocytic (GFAP, glial fibrillary acidic protein; Figure 1 D, H) and neuronal [microtubule associated protein-2 (MAP-2), synaptophysin; Figure 1 E, F, I, K] markers revealed normal appearance of astrocytes and normal synaptic and dendritic pattern of neurons. Also, vascularization of grafts of both genotypes appeared regular. All grafts showed a variable, perivascular lymphocytic infiltration, presumably due to mild graft rejection. These findings are well explained with slight histoincompatibility between graft and host tissue, and are consistent with earlier findings (Isenmann *et al.*, 1996).

Our results indicate that MTF-1 is dispensable for the maturation of CNS tissue, at least under non-stress conditions. However, although the host brain provides a good environment for differentiation into neurons, astrocytes and oligodendrocytes, even allowing for the formation of synapses and arrangements of neurons resembling the hippocampal ribbon, it is not suitable for the study of the structural organization of more complex functional and architectonic patterns of the CNS. Therefore, it remains to be seen whether MTF-1 is involved in such a developmental context.

In addition, it cannot be formally excluded that the surrounding host wild-type brain contributes to proper development of the grafted cells, either by supplying soluble factors, or by protecting the grafted tissue by scavenging stressors.

As discussed above, absence of functional MTF-1 reduces the downstream action of metallothioneins in modulating the concentration of free intracellular zinc. Since all grafts devoid of MTF-1 do not appear to suffer from neurotoxicity, it may be concluded that intracellular zinc levels do not reach toxic levels in such an environment.

This finding was not necessarily expected, considering that all metallothioneins are zinc-binding proteins, and that zinc is a well known neurotoxic agent (Yokoyama *et*

For paraffin histology, whole mouse brains were fixed in 4% paraformaldehyde in phosphate-buffered saline. Coronal slices were dehydrated through graded alcohols and embedded in paraffin. Sections of 3 mm nominal thickness were mounted on silanized glass slides and routinely stained with hematoxylin and eosin. Immunostains for glial fibrillary acidic protein [GFAP, polyclonal, 1:300 (DAKO, Glostrup, Denmark)], microtubule associated protein (MAP-2) (monoclonal, 1:1000, Boehringer Mannheim, Germany), synaptophysin (polyclonal 1:40, DAKO) were performed. Biotinylated, secondary antibodies (goat anti-rabbit; rabbit anti-mouse, DAKO) were used at a dilution of 1:200. Visualization was achieved using the biotin/avidin-peroxidase system (DAKO) with diaminobenzidine as a chromogen.

et al., 1986; Choi *et al.*, 1988; Lees *et al.*, 1990; Duncan *et al.*, 1992; Freund and Reddig, 1994), and its intracellular accumulation may contribute to neuronal death (Frederickson *et al.*, 1989; Weiss *et al.*, 1993).

While the MT-I and MT-II genes are most likely inactive in the MTF-1^{-/-} neural tissue, there is no further evidence from the above findings that MT-III is mainly regulated by MTF-1, since no effects of a potential MT-III downregulation can be seen in the MTF-1^{-/-} graft. Thus, the transcriptional dependence of the brain-specific MT-III gene on MTF-1 remains unclear, as MT-III contains potential metal responsive elements in its promoter sequence (Palmiter *et al.*, 1992), which in cell culture do respond to heavy metal load (S. Binder, O. Georgiev and W. Schaffner, unpublished results). However, MT-III expression failed to respond significantly to zinc or cadmium treatment of mice (Palmiter *et al.*, 1992).

Glutathione has been shown to be an important factor for the maintenance of the neuronal support function of astrocytes by providing glutathione to neurons; accordingly, in a state of glutathione deficiency, neurons are more vulnerable to oxidative damage (Cooper and Kristal, 1997; Drukarch *et al.*, 1997; Castagne and Clarke, 1997; Dringen and Hamprecht, 1998; Dringen *et al.*, 1999). Therefore it may be further argued that MTF-1-deficient neural tissue is not affected by the consequence of relatively decreased glutathione levels under physiological conditions.

The availability of mature CNS tissue devoid of functional MTF-1 may now facilitate the detailed analysis of oxidative and other stressors both *in vivo* and *ex vivo*. In particular, the role of stress-protecting factors acting downstream of MTF-1, among them the metallothioneins and gamma-GCS_{nc}, can now be assessed when hypoxic or ischemic injury or heavy metal ions are applied.

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